AD-A158 822 THE TOXICITY OF GUANIDINE NITRATE TO FRESHWATER AQUATIC ORGANISMS(U) ARMY MEDICAL BIOENGINEERING RESEARCH AND DEVELOPMENT LAB FORT DETRICK MD W H SCHALIE JUN 85 1/1

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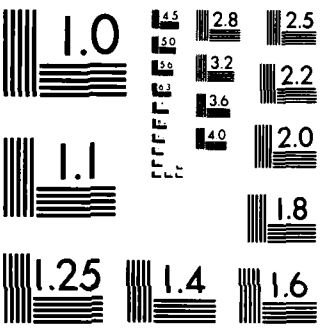

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TECHNICAL REPORT 8504

THE TOXICITY OF GUANIDINE NITRATE TO FRESHWATER AQUATIC ORGANISMS

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER TECHNICAL REPORT 8504	2. GOVT ACCESSION NO. AD-A158822	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)* THE TOXICITY OF GUANIDINE NITRATE TO FRESHWATER AQUATIC ORGANISMS		5. TYPE OF REPORT & PERIOD COVERED Technical Report Feb 1984 - Nov 1984 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) WILLIAM H. van der SCHALIE, Ph.D.		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS US Army Medical Bioengineering Research and Development Laboratory, ATTN: SGRD-UBG Fort Detrick, Frederick, MD 21701-5010		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62720A 3E162720A835/00/123
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, MD 21701-5012		12. REPORT DATE June 1985 13. NUMBER OF PAGES 27
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*		15. SECURITY CLASS. *(of this report)* UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Acute toxicity	Fish
Aquatic toxicology	Guanidine nitrate
Daphnia magna	Invertebrates
Fathead minnow	Pimephales promelas

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The toxicity of guanidine nitrate (GN) to fathead minnows (Pimephales promelas) and water fleas (Daphnia magna) was determined. Based on immobilization of D. magna, the static acute 48 hour EC50 of GN was 70.2 mg/L, with 95 percent confidence limits of 66.0 to 74.7. An initial 21 day flow-through chronic test with D. magna showed that significant toxic effects occurred at the lowest GN concentration tested, 4.2 mg/L. In a second chronic test, statistically significant effects on reproduction (young per female per

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reproductive day) were found at 6.09 mg/L, but no effects were found at 2.90 mg/L, the next lowest concentration. Fathead minnows exposed to GN in a 35 day early life stage test were much less sensitive than D. magna. Statistically significant effects on survival were noted at 424 mg/L but not at lower concentrations (181 mg/L and below). To allow better evaluation of the potential effects of GN on aquatic communities, more data should be obtained on the toxicity of GN to additional fish, aquatic invertebrate, and aquatic plant species. The effects of water quality parameters on toxicity and the toxicity of significant environmental breakdown products of GN (if any) should also be determined.

ACKNOWLEDGMENTS

The efforts of the many individuals at the US Army Medical Bioengineering Research and Development Laboratory (USAMBRDL) were necessary for the generation and analysis of the data described in this technical report. Statistical analyses of the chronic toxicity data were provided by Ms. F. Broski. Purification of the guanidine nitrate used in testing and analysis of aqueous test samples were provided by chemists including Dr. S. Hoke and Ms. L. Baxter. Toxicity tests were conducted by Mr. T. Shedd, Mr. R.C. Bishoff, and SP4 M. Skarwecki. Ms. M.F. Bostian helped in preparation of the manuscript.

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INTRODUCTION

Guanidine nitrate (GN) is an intermediate in the production of nitroguanidine at Sunflower Army Ammunition Plant (SAAP), DeSoto, Kansas. Since GN may be discharged in wastewater generated at SAAP, both state and federal regulatory authorities have requested additional information on the toxicity of GN to aquatic organisms. Specific tests requested include acute and chronic toxicity tests with the water flea Daphnia magna and an early life stage test with the fathead minnow, Pimephales promelas. The results of these tests are given in this report. The objective of the acute test with D. magna was to determine a 48 hour EC50, while the objectives of the daphnid chronic and fathead minnow early life stage tests were to provide an estimate of both a no observed effect concentration (NOEC) and a lowest observed effect concentration (LOEC) for GN. The NOEC is the highest concentration tested that does not cause statistically significant differences from the controls for any of the parameters analyzed, while the LOEC is the next higher concentration tested. All available data on the toxicity of GN to aquatic organisms are summarized.

METHODS AND MATERIALS

GN TEST SOLUTION PREPARATION AND ANALYSIS

GN was obtained from the Eastman Kodak Company, Rochester, New York. The lot number was A 8 B, with a melting point specified as a 2°C range including 213°C. This material was dissolved in methanol, filtered, recrystallized, and dried prior to use in testing. Test solutions were made from dilutions of stock solutions of GN dissolved in well water. Test solution concentrations of GN were measured by ion chromatography. Details of the analytical method, including precision and accuracy data for GN in distilled water, are described elsewhere.[1] Well water samples were analyzed as described in the reference, except that additional time was required between samples to allow for elution of calcium and magnesium ions. All samples were filtered through a 0.22-micron membrane filter just prior to analysis. All test concentrations are reported as guanidine nitrate. Spiked samples of GN were included with each test solution sample set submitted for analysis. The average spike recoveries are shown in Table 1.

DILUTION WATER QUALITY

The well water used in testing was obtained from a 62-meter well at Ft. Detrick. A water softening system was used that mixed softened and raw well water and reduced the hardness of the original well water by about 40 percent. This water was then pumped through a spray nozzle for aeration and sent through a 5-micron cellulose acetate cartridge filter, an ultraviolet sterilizer, and temperature control equipment prior to its use in testing.

TABLE 1. RECOVERY OF GN FROM SPIKED TEST SOLUTION SAMPLES

Test	Average Spike Recovery (%)	Standard Deviation (%)	Range (%)	Number of Samples
Daphnid Static Acute	94.1	± 1.1	93.3 - 94.8	2
First Daphnid Chronic	93.3	± 6.4	83.5 - 98.0	5
Second Daphnid Chronic	100.8	±12.5	89.4 - 118.0	6
Fathead Minnow Early Life Stage	103.3	± 7.9	96.4 - 121.0	8

A comprehensive analysis of the dilution water done during the period of testing is given in Table 2. A few heavy metals were present at very low concentrations. Chlorinated hydrocarbons were uniformly below detection limits. The good condition of control animals in the daphnid and fathead minnow tests is the best evidence that the dilution water quality was acceptable.

Test water quality conditions were fairly stable, as shown in Tables 3 to 6. Mean well water alkalinity, hardness, and conductivity values for each test varied less than 15 percent from the means for these parameters over all tests. For all test solutions measured in each test, the range in pH was no more than 0.5 pH units. Dissolved oxygen concentrations were greater than 80 percent of saturation in the daphnid tests and greater than 60 percent of saturation in the fathead minnow early life stage test. All fathead minnow test tanks were gently aerated after day 18 of the test. Test temperatures were within one degree of the target temperature except for one 10-hour period in the first daphnid chronic test, when temperatures fell to 1.7°C below the target temperature.

TEST METHODS

The D. magna used in testing were obtained from an in-house culture unit. For both holding and testing, daphnids were kept on a 16-hour light, 8-hour dark photoperiod under wide spectrum fluorescent bulbs (Durotest® Optima 50 bulbs, Color Rendering Index 91), with light intensities of about 200 lux (stock cultures) and 400 lux (test). During holding, stock daphnids were housed in 2 L glass tanks with 10 daphnids per tank. Aerated well water flowed through the tanks at a rate of two tank volumes per day. Temperature was maintained at 20°C (range 19° to 21°C). Daphnids were fed twice each day,

* Use of trademarked name does not imply endorsement by the US Army, but is used only to assist in identification of a specific product.

TABLE 2. COMPREHENSIVE DILUTION WATER ANALYSIS

Parameter	Concentration (mg/L) Sample 1	Sample 2
Ammonia (as N)	0.12	-[a]
Nitrite (as N)	<0.01	-
Nitrate (as N)	0.14	-
Chloride	70	-
Cyanide	0.037	-
Fluoride	0.22	-
Sulfate	43	-
Sulfide	<1	-
Total Organic Carbon	0.7	-
Aluminum	0.078	<0.002
Arsenic	0.0035	-
Barium	0.104	-
Boron	-	0.05
Cadmium	<0.001	-
Chromium	<0.002	-
Cobalt	0.0065	<0.002
Copper	0.0035	0.008
Iron	0.1	0.1
Lead	<0.002	-
Manganese	0.008	<0.002
Mercury	<0.0005	-
Nickel	<0.003	-
Phosphorus	0.01	-
Selenium	<0.002	-
Silver	<0.001	-
Zinc	0.05	0.04

Chlorinated Hydrocarbons Parameter	Concentration (µg/L)
Aldrin	<0.02
p,p'-DDT	<0.02
o,p'-DDT	<0.02
DDD	<0.02
DDE	<0.02
Dieldrin	<0.02
Endosulfan I	<0.02
Endosulfan II	<0.02
Endosulfan Sulfate	<0.02
Endrin	<0.02
Endrin Aldehyde	<0.02
Heptachlor	<0.02
Heptachlor Epoxide	<0.02
Lindane	<0.01
Alpha-BHC	<0.01
Beta-BHC	<0.02
Delta-BHC	<0.02
Methoxychlor	<0.20
Polychlorinated Biphenyls	<2.0
2,4-D	<0.06
2,4,5-T	<0.06
Silvex	<0.06
Diazinon	<0.02
Malathion	<0.10
Parathion	<0.10

a. Not measured.

TABLE 3. WATER QUALITY DURING THE DAPHNID STATIC ACUTE TEST

Parameter Monitored	Frequency; Point of Sampling	Results				Monitoring Method or Instrument
		Mean	Standard Deviation	Range	n	
Hardness (mg/L as $CaCO_3$)	Initially; well water	197	-	-	1	Reference 2
Alkalinity to pH 4.5 (g/L as $CaCO_3$)	Initially; well water	225	-	-	1	Reference 2
Specific Conductivity (µmhos/cm)	Initially; well water	868	-	-	1	Yellow Springs Instrument Model 32 Conductivity Meter
pH	0 and 48 hours; each treatment level	-	-	8.5-8.7	12	Cole Parmer Digi-Sense pH Meter
Dissolved Oxygen (mg/L)	0 and 48 hours; each treatment level	-	-	8.3-9.0	12	Yellow Springs Instrument Model 58 Oxygen Meter
Temperature (^{o}C)	Continuous; water bath	-	-	19.4-20.6	-	Cole Parmer Series 8354 Miniature Temperature Recorder
	0 and 48 hours; each treatment level	20.2	-	20.0-20.4	12	Cole Parmer Digital Thermometer

TABLE 4. WATER QUALITY DURING THE FIRST DAPHNID CHRONIC TEST

Parameter Monitored	Frequency; Point of Sampling	Results				Monitoring Method or Instrument
		Mean	Standard Deviation	Range	n	
Hardness (mg/L as $CaCO_3$)	Biweekly; well water	169	0	-	2	Reference 2
Alkalinity to pH 4.5 (mg/L as $CaCO_3$)	Biweekly; well water	214	± 4.2	211-217	2	Reference 2
Specific Conductivity (µmhos/cm)	Biweekly; well water	700	± 3.5	698-703	2	Yellow Springs Instrument Model 32 Conductivity Motor
pH	Weekly; each replicate tank	-	-	7.8-8.3	114	Cole Parmer Digi-Sense pH Meter
Dissolved Oxygen (mg/L)	Daily; each replicate tank	-	-	8.1-8.5	114	Yellow Springs Instrument Model 58 Oxygen
Temperature (^{o}C)	Continuously; water bath	-	-	18.3[a]-21.1	-	Cole Parmer Series 8354 Miniature Temp. Recorder
	Daily; two tanks	20.1	-	19.6-20.8	44	Cole Parmer Digital Thermometer

a. Water bath temperature was below 19°C for less than 10 hours on Day 17 of the test.

TABLE 5. WATER QUALITY DURING THE SECOND DAPHNID CHRONIC TEST

Parameter Monitored	Frequency; Point of Sampling	Results				Monitoring Method or Instrument
		Mean	Standard Deviation	Range	n	
Hardness (mg/L as $CaCO_3$)	Weekly; well water	173	± 7.3	165-181	4	Reference 2
Alkalinity to pH 4.5 (mg/L as $CaCO_3$)	Weekly; well water	232	± 9.0	221-242	4	Reference 2
Specific Conductivity (μmhos/cm)	Weekly; well water	683	± 37.8	628-709	4	Yellow Springs Instrument Model 32 Conductivity Motor
pH	Weekly; each replicate tank	-	-	7.8-8.2	125	Cole Parmer Digi-Sense pH Meter
Dissolved Oxygen (mg/L)	Daily; each replicate tank	-	-	7.6-8.7	125	Yellow Springs Instrument Model 58 Oxygen
Temperature (^{o}C)	Continuous; water bath	-	-	19.0-20.8	-	Cole Parmer Series 8354 Miniature Temp. Recorder
	Daily; two tanks	20.1	-	19.4-20.6	40	Cole Parmer Digital Thermometer

TABLE 6. WATER QUALITY DURING THE FATHEAD MINNOW EARLY LIFE STAGE TEST

Parameter Monitored	Frequency; Point of Sampling	Results				Monitoring Method or Instrument
		Mean	Standard Deviation	Range	n	
Hardness (mg/L as $CaCO_3$)	Weekly; well water	189	± 12.3	169-197	5	Reference 2
Alkalinity to pH 4.5 (mg/L as $CaCO_3$)	Weekly; well water	219	± 7.7	211-226	5	Reference 2
Specific Conductivity (µmhos/cm)	Weekly; well water	850	± 33.1	795-875	5	Yellow Springs Instrument Model 32 Conductivity Meter
pH	Weekly; each replicate tank	-	-	8.2-8.6	71	Cole Parmer Digi-Sense pH Meter
Dissolved Oxygen (mg/L)	Daily; each replicate tank	-	-	5.2-8.5	393	Yellow Springs Instrument Model 58 Oxygen Meter
Temperature (^{o}C)	Continuously; water bath	-	-	24.7-25.8	-	Cole Parmer Series 8354 Miniature Temperature Recorder
	Daily; each replicate tank	25.2	-	24.8-25.8	343	Cole Parmer Digital Thermometer

7 days a week with Ankistrodesmus falcatus raised and supplemented with vitamins as recommended by Goulden et al.[3] Feeding levels were approximately 2 mg/L (dry weight) in the morning and 4 mg/L in the afternoon. Newly-released daphnids were removed from the culture tanks and discarded every Monday, Wednesday, and Friday. To obtain neonate daphnids for testing, all non-adult daphnids were removed from the stock tanks less than 24 hours prior to the start of the test. On the day of the test, newly-released daphnids from each stock tank were transferred (using an eyedropper having a fire-polished bore at least 2 mm in diameter) into a beaker of well water. Neonates were obtained from 27 day-old daphnids for the static acute test, 11 day-old daphnids for the first daphnid chronic test, and 32 day-old daphnids for the second daphnid chronic test.

After the daphnids for the GN static acute test were obtained, they were transferred into a random sequence of test chambers (250 mL borosilicate beakers containing 200 mL of test solution) in groups of 3, 3, and 4 until each beaker contained 10 daphnids. The transfer pipette was rinsed between each transfer. There were three replicate beakers (30 daphnids) per treatment level. The test beakers were placed in a 20°C water bath in a randomized pattern.

At the beginning of the static acute test, pH, dissolved oxygen, and temperature were measured in the test solutions at each treatment level prior to the distribution of the solution to the test beakers. Samples for GN analysis were also taken at that time. At the end of the test, these same analyses were done for one replicate beaker at each treatment level. Any floating daphnids were removed from the surface film after 24 hours of exposure by gently dropping test solution on them. After 24 and 48 hours of exposure, the number of immobilized daphnids in each beaker was recorded. Immobilization was defined as the complete absence of movement following gentle agitation of the beaker. A 48 hour EC50 based on immobilization was determined using the trimmed Spearman-Karber method.[4,5]

The daphnid chronic tests were conducted under flow-through conditions in well water. Four replicates with 10 daphnids each were exposed to GN at each of six treatment levels, including controls. The test was started with less than 24-hours-old daphnids and lasted for 21 days. End points monitored included survival, reproduction, and growth. Test solutions were delivered to the test tanks using a quarter-liter proportional diluter constructed as described by Lemke et al.,[6] except that no neoprene stoppers were used, and glass-glass connections were made with the aid of nothing other than silicone glue or heat-shrinkable perfluorocarbon tubing. The diluter cycle time was controlled by a timer that opened a solenoid valve to the dilution water. The dilution water flowed into the diluter until it made contact between two stainless steel electrodes in the last dilution water chamber, which resulted in the closing of the solenoid valve. The timer was set to deliver three cycles per hour. GN stock solution was delivered to the diluter's toxicant mixing chamber by a peristaltic pump, which was turned on for 1 minute every 20 minutes (between diluter cycles) by a Chrontrol^{R} programmable timer. The diluter provided each test tank with about two tank volumes of test solution per day. Test solutions leaving the test tanks were collected and passed through a deionization cartridge to remove GN. No GN was detected in the effluent from the cartridge.

Neonate daphnids were obtained as described above and then were randomly transferred in groups of three and four into 50 mL beakers containing about 25 mL of well water, until each beaker held 10 daphnids. The daphnids were then released into the test tanks. Rows of four replicate test tanks at each treatment level were arranged in a random sequence in the water bath. Test daphnids were raised in the same tanks as the stock daphnids, except that an exterior standpipe was added to raise the water level in the tank above the drain screen (286 micron polyethylene mesh). Each tank was a 14-centimeter cube with a drain at a height of 10 cm; the total test solution volume was 2.0 liters.

Toxicant concentrations were measured weekly in at least one replicate of each treatment level. The dissolved oxygen and pH levels were measured in one replicate of each treatment every day. Water bath temperature was monitored continuously, while test tank temperature was checked in two tanks every day. A submersible circulating pump was used in the water bath to help ensure uniform temperatures. Test daphnids were fed as described above for the stock daphnids. Survival was noted daily in each test tank. After young were released in the tanks, young production was monitored every Monday, Wednesday, and Friday. On these days, adults were transferred into a beaker of test solution, and the test solution remaining in the test tank was poured through a brine shrimp net which retained the neonate daphnids. The test solution and the adults were returned to the test tank, and the neonates were rinsed into a beaker for counting. At the end of the test (day 21), surviving daphnids were anesthetized in a beaker containing 150 mL well water plus 5 mL of acetone and 0.2 mL quinaldine. The lengths of these daphnids (from the apex of the carapace to the base of the caudal spine) were measured with a stereoscope equipped with a calibrated ocular micrometer.

The following end points were analyzed statistically: survival, total young per replicate tank, young per female per reproductive day (total young divided by the sum of the total days that each daphnid was alive after the onset of reproduction in the test tank), and growth (length). Evaluation of the survival data was done by methods described in Feder and Collins.[7] Initial chi-square tests for homogeneity of variance within treatments were conducted. The odds ratio test was used to determine the relative risk of immobilization occurring at a GN treatment level, relative to the controls. Pairwise comparisons were done between controls and each GN treatment to determine statistically significant differences. Corrections for simultaneity were done according to Bonferonni's method.[7]

For the remaining non-quantal variables, the General Linear Models program of the Statistical Analysis System[8] was used to generate a quadratic regression model for response vs. concentration (logarithmically transformed). From this model, concentrations of GN eliciting a given response (e.g. an EC50) could be determined. Pairwise tests for significant differences between the controls and each treatment group were done by means of t-tests. Bonferroni's correction for simultaneity was applied.

The fathead minnow early life stage test was conducted under flow-through conditions in well water. Two replicates of 45 fathead minnows each were exposed to GN at each of six treatment levels, including controls. The test was started with embryos less than 24 hours old and lasted for 35 days.

Fathead minnow eggs were obtained from an in-house culture unit. Adult fathead minnows used for breeding were held at 25°C in aquaria containing spawning substrates. The substrates were sections of 10 cm PVC pipe cut in half lengthwise, with polyethylene liners to facilitate the removal of eggs. Spawning fathead minnows were fed frozen brine shrimp (Artemia salina) and Rangens No. 3 trout food (Fish and Wildlife Service formulation). Lighting conditions were the same as those used for the daphnids, except that light intensity during testing was about 150 lux.

Clean spawning substrate liners were placed in each substrate less than 24 hours prior to the start of the test. On the day of the test, 723 eggs were obtained from four substrates and were removed from the liners by gently rolling them with a fingertip. The eggs were pooled and examined under a stereoscope; any unfertilized or damaged eggs were discarded. Eggs (either individual or in clumps of two) were randomly transferred five at a time into numbered egg cups in beakers of well water until each cup held 45 eggs. The egg cups were 11.5 cm lengths of 50 mm ID glass tubing covered at the bottom with 506 micron polyethylene mesh screen.

After randomization, one egg cup was transferred to each of the 12 test tanks (two replicates of five treatment levels plus a control). The egg cups were kept in constant motion by a rocker-arm apparatus. Vertical travel was about 2 cm at a speed of 5 cycles per minute. The test tanks themselves were 9.4 L aquaria containing 6.9 L of test solution. The tank drains were covered with 506 micron mesh polyethylene screen to prevent loss of the fry. The test tanks were situated in a 25°C water bath; temperature was controlled by heating and cooling units. Test tanks were arranged in two rows of six in the water bath, and treatments were randomly assigned to tanks.

Toxicant solutions were delivered to the test tanks by a half-liter diluter system similar to the quarter-liter system described above for the daphnid chronic tests. The diluter cycled once an hour, and the peristaltic pump which delivered the GN stock solution was turned on for 2 minutes every hour between diluter cycles by a Chrontrol® programmable timer. The diluter provided each test tank with about 0.9 tank volumes of test solution per day. A higher flow rate was not selected because it would have required an enormous amount of GN; nearly 400 g was required to conduct the present study. GN was removed from the test solutions flowing out of the test tanks by using deionization cartridges as described for the daphnid chronic tests.

After the start of the test, the egg cups were removed and examined daily, and dead eggs were removed. When egg hatch was nearly completed, fry were allowed to swim out of the egg cups into the test tank itself. Any remaining unhatched eggs were left in the egg cups until they either hatched or died. Fry were fed twice daily with newly hatched brine shrimp nauplii. The brine shrimp cysts used came from Colombia, South America. Excess food and fecal materials were siphoned from each tank daily, or as needed. Fry were not fed for the last 24 hours of the test to allow their guts to empty prior to weighing.

The following end points were monitored during the test: time to hatch, egg hatching success, fry survival, overall survival, growth, and deformities. Time to hatch is the number of days from the start of the test required for hatching of at least 50 percent of the eggs that eventually do hatch.

Overall survival includes both embryo survival (egg hatching success) and fry survival. The growth of each surviving fish was measured at the end of the test. Measurements included both standard length and weight (blotted dry). Deformities were noted for dead fry and for those that survived to the end of the test. Statistical analyses were the same as those described above for the daphnid chronic tests. Time to hatch was not analyzed statistically.

RESULTS AND DISCUSSION

Daphnids exposed to GN during the static acute test showed a tendency to be caught in the well water surface film (Table 7). Although there is not a clear concentration-response relationship, the floating is thought to represent a toxicant effect, since no floating daphnids were found in the controls. The 48 hour EC50 computed from this data was 70.2 mg/L with 95 percent confidence limits of 66.0 to 74.7.

TABLE 7. RESULTS OF THE DAPHNID 48 HOUR STATIC ACUTE GN TEST

Nominal GN Concentration (mg/L)	Mean Measured GN Concentration[a] (mg/L)	Percent Immobilization[b] 24 Hours		48 Hours	
100	97.5	6.7	(33.3)[c]	100	(-)
60	58.5	0	(10.0)	13.3	(3.3)
36	40.5	0	(26.7)	3.3	(16.7)
21.6	25.0	0	(13.3)	0	(6.7)
13.0	14.5	0	(20.0)	0	(3.3)
0 (Control)	<0.5[d]	0	(0)	0	(0)

a. Mean of two measurements.
b. Percent of 30 daphnids exposed at each treatment level.
c. Percent of daphnids caught in surface film.
d. Below detection limit.

Measured concentrations in the first daphnid chronic test (Table 8) averaged 18 percent below nominal levels. The toxicant diluter functioned well; the only deviation from normal operation was a 5-hour shutdown on day 19 of the test, during which no test solutions flowed into the tanks. Overall results from the chronic test are given in Table 9. The only concentration with significantly higher immobilization than the controls was 52.0 mg/L,

where all daphnids were affected. (The 16.8 mg/L treatment level could not be included in this analysis due to heterogeneity among replicates, but overall immobilization in this treatment was only 5 percent, with two replicates having 0 percent and two replicates having 10 percent immobilization.)

TABLE 8. MEASURED GN CONCENTRATIONS - FIRST DAPHNID CHRONIC TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
60	52	-	-	1
33.6	26.2	± 0.87	25-28	4
18.8	16.8	± 2.87	15-21	4
10.5	8.8	± 0.50	8-9	4
5.9	4.2	± 2.06	2-7	4
0	<1[a]	-	-	4

a. Below detection limit.

TABLE 9. RESULTS OF THE FIRST DAPHNID CHRONIC TEST WITH GN

Mean Measured Concentration (mg/L)	Immobilization[a] (%)	Young per Female Per Reproductive Day[b]	Total Young Per Tank[b]	Length[c] (mm)
52.0	100[d]	-	-	-
26.2	22.5	1.02[d]	83.8[d]	2.89[d]
16.8	5.0	2.91[d]	331.5[d]	3.40[d]
8.8	7.5	4.24[d]	468.8[d]	3.78[d]
4.2	12.5	6.22[d]	683.5[d]	4.18[d]
<1 (control)	12.5	7.93	914.0	4.51

a. Total for four replicate tanks at each treatment level
b. Mean of four replicate tanks at each treatment level.
c. Mean length per daphnid for four replicate tanks at each treatment level.
d. Significantly different from the controls (overall $\alpha = 0.05$).

Both reproduction (young per female per reproductive day and total young) and growth (length) were significantly reduced from the controls at all concentrations tested. At the lowest concentration tested (4.2 mg/L), young per female per reproductive day and total young were reduced from control levels by 22.2 percent and 25.8 percent, respectively, while length was reduced 7.3 percent. A NOEC could not be defined from this study since statistically significant effects of GN were noted at the lowest concentration tested, 4.2 mg/L. A second daphnid chronic test was therefore conducted.

The top test concentration in the second chronic test was set at about 6 mg/L, which should have caused substantial toxic effects based on the results from the first test. Actual test concentrations are given in Table 10. No diluter malfunctions were encountered during the test. Since the detection limit of the GN analysis method was 1 mg/L, the measured concentrations at the lowest treatment level (0.63 mg/L nominal) must be considered to be estimates. The biological data at this concentration did not influence the overall conclusions for the test.

TABLE 10. MEASURED GN CONCENTRATIONS - SECOND DAPHNID CHRONIC TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
6.19	6.09	± 0.179	5.94-6.35	4
3.45	2.90	± 0.143	2.77-3.05	4
1.97	1.66	± 0.140	1.55-1.84	4
1.08	1.14	± 0.162	1.04-1.38	4
0.63	0.75[a]	± 0.0520	0.72-0.81	3
0	<1[b]	-	-	4

a. Estimated concentration.
b. Below detection limit.

The only statistically significant reduction from the controls was a 12 percent decrease in young per female per reproductive day at 6.09 mg/L (Table 11). A 10 percent reduction in total young at this concentration was close to being significantly different from the controls; the actual p value was 0.025 but using Bonferonni's correction, a p value of 0.01 is required to meet an overall significance level of $\alpha = 0.05$. A slight (2 percent) but statistically significant increase in length at the lowest concentration was also found. GN was much more toxic to daphnids in the first daphnic chronic test than in the second. Based on the quadratic regression model for young per female per reproductive day from the first test, a 32 percent reduction from the controls would have been predicted at the top concentration in the

TABLE 11. RESULTS OF THE SECOND DAPHNID CHRONIC TEST WITH GN

Mean Measured Concentration (mg/L)	Immobilization[a] (%)	Young per Female Per Reproductive Day[b]	Total Young Per Tank[b]	Length[c] (mm)
6.09	0	9.32[d]	1,305	5.01
2.90	0	10.2	1,432	5.12
1.66	0	10.7	1,499	5.17
1.14	5.0	10.5	1,397	5.23
0.75	7.5	10.6	1,400	5.24[d]
<1 (control)	0	10.6	1,453	5.11

a. Total for four replicate tanks at each treatment level
b. Mean of four replicate tanks at each treatment level.
c. Mean length per daphnid for four replicate tanks at each treatment level.
d. Significantly different from the controls (overall $\alpha = 0.05$).

second test (6.09 mg/L GN), but only a 12 percent reduction was observed. Daphnids used in the first test may have been more sensitive to GN because they were taken from adults that were only 11 days old, and first brood daphnids could have been included among the test organisms. First brood daphnids have been observed to be smaller and weigh less than daphnids of subsequent broods,[3] and they could well be more sensitive to toxicants.

The NOEC to LOEC range derived from the second daphnid chronic test is 2.90 to 6.09 mg/L. In a 21 day GN daphnid chronic test conducted by Cooney et al.,[9] the NOEC to LOEC range (measured concentrations) was 4.81 to 9.95 mg/L, with time to first reproduction being the most sensitive parameter tested. (Time to first reproduction in the second daphnid test was 8 to 9 days in all treatments.)

The relationship between nominal and measured GN concentrations for the fathead minnow early life stage test is given in Table 12. The mean measured concentrations were fairly close to the nominal levels, although the variability at 200, 50, and 25 mg/L (nominal) was relatively high. A minor diluter malfunction on day 20 of the test resulted in an average 10 percent reduction in test concentrations.

TABLE 12. MEASURED GN CONCENTRATIONS DURING THE FATHEAD MINNOW EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
400	424	± 23.5	380-460	14
200	181	± 26.8	155-253	17
100	98.1	± 9.6	86-117	13
50	52.7	± 9.8	36-72	13
25	27.3	± 7.0	15-40	14
0	<0.5[a]	-	-	12

a. Below detection limit.

Test results are summarized in Table 13. Statistical analysis revealed no evidence of tank to tank heterogeneity for any parameter monitored. Statistically significant GN-related effects occurred only at 424 mg/L, where there was nearly complete fry mortality. Acute mortality (within 96 hours of hatch completion) accounted for about three-fourths of the mortality at this concentration. Odds ratio analysis showed that fish exposed to 424 mg/L were 192 times more likely than a control fish to die after hatching (95 percent confidence limits 46 to 1,111 times) and 116 (31 to 625) times more likely to die overall. There were no statistically significant differences from the controls at any treatment level for hatching success, fry deformities, or fry growth. Variability in control growth was high; the relative standard deviation for control weight was 50 percent. However, mean fry lengths and weights (Table 13) show no evidence of a concentration-related trend. No behavioral effects of GN exposure were noted at any concentration except the highest. At this level, the fish were sensitive to disturbances, swimming much more rapidly than the other fish when their tank was cleaned or when food was added. No loss of equilibrium was observed. Based on these data, the NOEC to LOEC range is 181 to 424 mg/L.

Available toxicity data for GN and freshwater aquatic organisms are summarized in Table 14. The reported 48 hour EC50 for D. magna of 23 mg/L may have been low because this test was conducted with daphnids obtained from yeast-fed adults. Non-algal fed daphnids may be in poorer condition[3] and thus more sensitive to toxicant than those provided algae. Neonates used in the other three daphnid acute toxicity tests were obtained from adults fed either solely on algae or on algae plus a trout chow supplement. The limited acute toxicity data available for fish suggest that fish are substantially less sensitive to GN than are daphnids.

TABLE 13. RESULTS OF THE FATHEAD MINNOW EARLY LIFE STAGE TEST WITH GN

Mean Measured Concentration (mg/L)	Time to Hatch[a] (Mean Days to 50% Hatch)	Hatching Success (%)	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Length (mg)
424	4.5	83.3	4.0[b]	3.3[b]	2.7	13.7	48
181	4	83.3	93.3	77.8	2.7	14.4	46
98.1	4	87.8	86.1	75.6	1.3	14.7	49
52.7	4	87.8	86.1	75.6	0.0	14.4	47
27.3	4	88.9	80.0	71.1	0.0	14.6	48
<0.5 (control)	4	90.0	88.9	80.0	0.0	14.6	48

a. Not analyzed statistically.
b. Significantly different from the controls (overall $\alpha = 0.05$).

TABLE 14. SUMMARY OF THE TOXICITY OF GN TO AQUATIC ORGANISMS

Species[a]	Test Type	Length of Exposure (days)	Test Temp. (°C)	Test End Point	Results (mg/L)[b]	Reference
Ictalurus punctatus (Channel catfish)	Flow-Through Acute	4	20-22	LC50	1,850	10
Pimephales promelas (Fathead minnow)	Flow-Through Acute	4	20-22	LC50	690 (535-890)	10
P. promelas	Early Life Stage	35	25	NOEC-LOEC	181-424	This Report
Daphnia magna	Static Acute	2	20	EC50	70.2 (66.0-74.7)	This Report
Daphnia magna	Static Acute	2	20	LC50	59.3[c,d] (32-100)	11
Daphnia magna	Static Acute	2	20	LC50	56.6[d]	9
Daphnia magna	Static Acute	2	17	LC50	23 (11-35)	10
Daphnia magna	Chronic	21	20	NOEC-LOEC	4.81-9.95	9
Daphnia magna	Chronic	21	20	LOEC	4.2[e]	This Report
Daphnia magna	Chronic	21	20	NOEC-LOEC	2.90-6.07	This Report

a. Tests arranged from highest to lowest end point concentrations.
b. The 95% confidence limits are reported, if available.
c. Mean of three LC50s (range 56-64 mg/L); confidence limits were the same for each LC50.
d. Based on nominal GN concentrations.
e. Lowest concentration tested.

Acute to chronic toxicity ratios (ACRs) may be calculated by dividing the acute EC50/LC50 by the geometric mean of the NOEC and LOEC values for both daphnids and fathead minnows. When computed in this way, the ACR for fathead minnows (using an LC50 and early life stage test from two different sources) is 2.5, while the ACR's for the two sets of daphnid tests are 16.7 (this report) and 8.2.[9]

A computer literature search of a variety of data bases (Table 15) provided very little additional information on the toxicity of guanidine to aquatic organisms. The toxicity of guanidine to mammals appears to be rather low; the oral lethal dose for rabbits was reported to be 500 mg/kg.[12]

TABLE 15. DATA BASES SEARCHED FOR GN AQUATIC TOXICITY INFORMATION

Aqualine
Aquatic Sciences and Fisheries Abstracts
Biological Abstracts
Life Sciences Collection
Medline
Merck Index
National Technical Information Service
Oceanic Abstracts
Register of the Toxic Effects of Chemical Substances
Waternet
Water Resources Abstracts

It is likely that the nitrate anion contributes little to the toxicity of GN to D. magna, but may be more significant for the toxic effects of GN on fish. A range-finding test done in this laboratory found the 48-hour EC50 for nitrate and D. magna to be 3,972 mg/L NO_3-N[13]. Since GN is about 11.5% NO_3-N, the highest LC50 value reported for D. magna (70.2 mg/L) would contain only about 8.1 mg/L nitrate-nitrogen. For fish, the only toxicity data found for nitrate was a 72-hour LC50 for guppies (Poecilia reticulatus) of 199 mg/L NO_3-N[14]. This corresponds to an equivalent GN concentration of 1,730 mg/L, which is not much different from the reported 96-hour GN LC50 for channel catfish. However, if the toxicity of nitrate to channel catfish is much less than it is to guppies, nitrate may still contribute little to GN toxicity in the channel catfish.

CONCLUSIONS

Daphnia magna appears to be much more sensitive to the acute and chronic effects of GN than are fathead minnows. Acute EC50s for daphnids are at least an order of magnitude below the LC50s for two fish species (Table 14). In addition, chronic toxic levels of GN for daphnids are roughly 10 to 20 times below the acutely toxic levels, while the chronic toxic levels for fathead minnows are estimated to be only 2.5 times below the acutely toxic level. The lowest concentrations of GN observed to cause toxic effects for any of the aquatic organisms tested were in the second daphnid chronic test, where the NOEC to LOEC range was 2.90 to 6.09 mg/L. Nitrate probably contributes little to the toxicity of GN to D. magna, but may be more significant to the toxicity of GN to fish.

Considerable caution should be used in drawing conclusions from these data concerning the potential effects of GN on aquatic communities, since the toxicity data base includes only two species of fish and one species of aquatic invertebrate. Additional data should be obtained concerning the acute and chronic toxicity of GN to other fish species, aquatic invertebrates (e.g. benthic invertebrates) and at least one algal or aquatic plant species. Additional studies on the toxicity of nitrate to aquatic organisms should be conducted to confirm the extent of contribution of this anion to overall GN toxicity. Acute tests to evaluate the effect of common water quality parameters on toxicity should also be conducted. Finally, the environmental fate of GN should be determined so that the toxicity of significant breakdown products can be evaluated.

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